JOURNAL OF BACTERIOLOGY, July 1982, p. 472–476
0021-9193/82/070472-05$02.00/0

Vol. 151, No. 1

Several Copies of the Same Insertion Sequence Are Present in Alpha-Hemolytic Plasmids Belonging to Four Different Incompatibility Groups

JUAN C. ZABALA, FERNANDO DE LA CRUZ, AND JOSÉ M. ORTIZ

Departamento de Bioquímica, Facultad de Medicina, Polígono de Cazoña s/n, Santander, Spain

Received 7 December 1981/Accepted 10 March 1982

Alpha-hemolytic plasmids belonging to four different incompatibility groups contained several copies (two to six) of the same DNA sequence. This sequence was capable of illegitimate recombination and thus behaved as an insertion sequence element. Its size (1.9 kilobases) and restriction enzyme cleavage map suggested that it was different from previously described insertion sequence elements.

Transposable elements are sequences of DNA that are able to promote a number of illegitimate recombination events. In *Escherichia coli*, they can act in the absence of a functional Rec system. Insertion sequence (IS) units are defined as transposable elements that do not encode any known phenotypically identifiable proteins (10).

The genetic information required for the production of the toxin α-hemolysin of *E. coli* resides in a 5-kilobase (kb) segment of DNA which is present in plasmids of different incompatibility groups (Hly plasmids) (5, 6, 12) that share a wide range of homology in their DNA sequences (7). Hly plasmids can undergo genetic recombination leading to recombinants containing different portions of the parental plasmids, but the recombination process is not understood and, particularly, no discrete transposable elements have been found (13). We report here the occurrence of a 1.9-kb IS element, which we call IS-Hly, in one Hly plasmid and the presence of similar DNA sequences in other Hly plasmids.

The ability of different Hly plasmids to mobilize the chloramphenicol resistance (Cm^r) marker of plasmid pACYC184 (2) was analyzed. Plasmid pACYC184 lacks the proteins necessary to promote its own mobilization and the site at which these proteins act (15); furthermore, it does not contain any sequence homologous to those of the Hly plasmids used in this work, as detected by Southern hybridization (5). Thus, conjugal transmission of pACYC184 must be brought about by its physical linkage with the tra^+ Hly plasmids. This form of transmission has been called conduction (3) and is thought to be promoted by transposable elements by means of the formation of cointegrates between the plasmids involved. Table 1 shows the results of such conduction experiments. All four Hly plasmids tested were able to promote the transfer of pACYC184 at frequencies of 10^{-6} to 10^{-7} per transconjugant plasmid. This cotransfer of pACYC184 and Hly plasmids was equally efficient from a *recA* background (strains SU105 and SU106), ruling out the involvement of homologous recombination.

Strain SU107 was one of the transconjugants from the mating SU102 × UB1636. When analyzed on agarose gels, strain SU107 showed three plasmid species of 102, 96, and 6.0 kb; the 96-kb band showed a lower relative intensity (data not shown). When UB5201 was transformed to Cm^r with DNA obtained from SU107, the 6.0-kb plasmid was obtained. Figure 1 shows the restriction map of this plasmid (pSU234). It shows an insertion of 1.9 kb within the 4.1-kb plasmid pACYC184. When SU107 was mated with UB5201, two types of transconjugants could be obtained. About 5% were Hly^+ but Cm^s and contained a plasmid of 96 kb; its restriction patterns for enzymes *Eco*RI and *Hin*dIII were identical to those of the original pSU233 (whose pattern is shown in Fig. 1). The majority of the transconjugants were Cm^r Hly^+ and contained the 102-kb plasmid (pSU235). Its restriction patterns with enzymes *Sal*GI, *Bam*HI, and *Hin*dIII are shown in Fig. 1. In each of the digestions, a single band originally present in pSU233 was missing and was replaced by two extra bands in pSU235. Plasmid pSU235 was Cm^r Tc^r, as well as Hly^+; thus, its restriction patterns and phenotype were consistent with it being a recombinant of plasmids pSU233 and pACYC184. The amount of extra DNA in pSU235, as calculated from the *Sal*GI digestion pattern, was about 6.1 kb (Fig. 1). As pACYC184 is only 4.1 kb, there is about 2.0 kb of DNA whose origin is not clear. This sequence could represent the duplication of the insertion sequence isolated in pSU234 (1.9 kb; see below). When plasmid DNA was prepared after repeated

TABLE 1. Conduction of plasmid pACYC184 by Hly plasmids of different incompatibility groups[a]

Donor strain[b]		Relevant characteristics[c] of the Hly plasmids	Frequency of Cm^r/Hly^+ exconjugants (10^{-7})[d]
Designation	Composition		
SU101	C600(pSU105 + pACYC184)	Hly^+ Tra^+ IncFVI, 123 kb	1
SU102	C600(pSU233 + pACYC184)	Hly^+ Tra^+ Inc(pSU233), 96 kb	10
SU103	C600(pSU316 + pACYC184)	Hly^+ Tra^+ IncFIII/IV, 77 kb	5
SU104	C600(pHly152 + pACYC184)	Hly^+ Tra^+ IncI2, 67 kb	0.8
SU105	UB5201(pSU233 + pACYC184)	Hly^+ Tra^+ Inc(pSU233), 96 kb	10
SU106	UB5201(pSU316 + pACYC184)	Hly^+ Tra^+ IncFIII/IV, 77 kb	10

[a] The term "conduction" is used in accordance with Clark and Warren (3). Donor strains were grown for 4 days at 30°C on nutrient agar plates. They were then mixed with approximately the same number of cells (~10^8) of the recipient strain UB1636 (*trp lys his* F^- Sm^r *E. coli* K-12) and spread on membrane filters, and the filters were incubated for 6 h at 37°C on the surface of nutrient agar plates. The cells were then suspended in broth, and suitable dilutions were plated on the indicated selection media.

[b] Strains SU101, SU102, SU103, and SU104 are derived from strain C600 (F^- *thr leu thi lac*). Strain C600 was first transformed with plasmid pACYC184 (2). Subsequently, the Hly plasmids pSU105, pSU233, pSU316, and pHly152 originally present in strain EC185 (3; F^-, prototroph, nalidixic acid resistant) were conjugated into C600(pACYC184), giving rise to strains SU101, SU102, SU103, and SU104, respectively. Strains SU105 and SU106 were constructed in the same way, except that the host strain was UB5201 (F^- *pro met recA56*).

[c] All of the Hly plasmids have been described (6) with the exception of pHly152 (11). Inc(pSU233) means that this plasmid was compatible with all prototypes tested (6).

[d] Expressed as the number of Cm^r transconjugants per Hly^+ transconjugant. The limit of detection of the experiment was about 5×10^{-9}. Selection plates contained 25 μg of chloramphenicol and 300 μg of streptomycin sulfate per ml for the Cm^r transconjugants and 5% washed horse blood and 300 μg of streptomycin sulfate per ml for the Hly transconjugants.

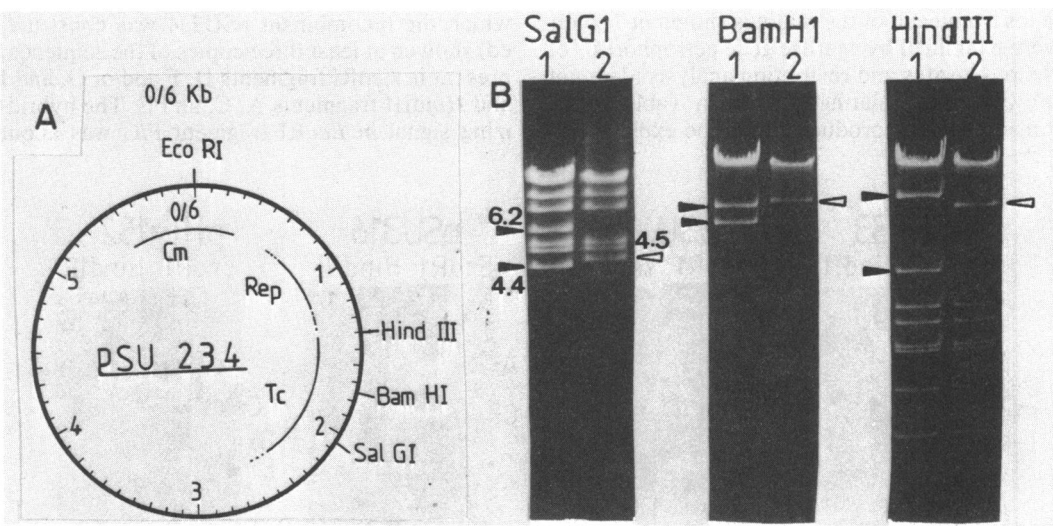

FIG. 1. Characterization of the plasmids present in strain SU107. (A) Restriction enzyme map of plasmid pSU234. Plasmid DNA was obtained from strain UB5201(pSU234) by centrifugation of Triton X-100-cleared lysates of that strain in cesium chloride-ethidium bromide density gradients (7) and mapped by analysis after single and double digestions with restriction enzymes *Eco*RI, *Hin*dIII, *Sal*GI, *Bam*HI, and *Pst*I. Plasmid pACYC184 contains single sites for *Eco*RI, *Hin*dIII, *Bam*HI, and *Sal*GI. As pSU234 is Cm^r (the *cat* gene in pACYC184 is located around the *Eco*RI site) and Tc^r (the *tet* genes of pACYC184 lie across the *Hin*dIII, *Bam*HI, and *Sal*GI sites), and the arrangement of the *Eco*RI, *Hin*dIII, *Bam*HI, and *Sal*GI sites in pSU234 is the same as in pACYC184, we can conclude that the sequence inserted in pACYC184 to produce pSU234 must be represented by a 1.9-kb DNA segment that lies between coordinates 2.5 and 5.5 kb in the pSU234 map. (B) *Sal*GI, *Bam*HI, and *Hin*dIII restriction patterns of plasmids pSU235 (tracks 1) and pSU233 (tracks 2). Plasmid DNA was obtained as described above from strains UB5201(pSU235) and UB5201(pSU233) and digested with the relevant restriction enzyme for 1 h at 37°C. The products of such digestions were run in 1.0% agarose gels in the presence of ethidium bromide (0.5 μg/ml) as described previously (5). The white arrows show the restriction fragments missing in pSU235 but originally present in pSU233. The black arrows show the two new fragments appearing in the recombinant DNA. The sizes of the fragments involved in the recombination could be calculated with good approximation only in the *Sal*GI digestion. Sizes are expressed in kilobases.

growth of strain UB5201 (pSU235), only the 102-kb plasmid species was observed. Thus, it can be assumed that plasmid pSU235 is stable in the *recA* background of strain UB5201.

The weak presence of the 96-kb plasmid in strain SU107 can be explained by assuming that pSU235 and pSU233 are incompatible, and thus pSU233 tends to be eliminated when selection pressure (Cm^r) is applied in the construction of SU107.

When the products of the mating SU105 × UB1636 were analyzed, results similar to those described for SU102 × UB1636 were found. Thus, the *rec* character of the donor did not affect the final structure of the conjugation products.

We assume from these data that the three plasmid species present in strain SU107 can be explained in the following way: transposition of the IS present in pSU233 to pACYC184 produces the cointegrate plasmid pSU235, which can then be transferred by conjugation. Plasmid pSU235 is an unstable cointegrate in the $recA^+$ background of strain UB1636 and breaks down, producing pSU234 and pSU233 as resolution products. Six to twenty-four transconjugant colonies from each of the matings shown in Table 1 were examined by agarose gel electrophoresis of cleared lysates and restriction analysis (data not shown). In all matings shown in Table 1, the transconjugants produced could be explained in the same way as for SU107. However, many of the pACYC184 recombinants were much larger than pSU234. Presumably, they were produced by resolution of the cointegrates across any of several different copies of the repeated sequences present in the Hly plasmids (see below; J. C. Zabala, F. de la Cruz, and J. M. Ortiz, manuscript in preparation).

Plasmid pSU234 was transcribed in vitro, and the labeled RNA transcripts were hybridized to restriction fragments of pSU233, pSU105, pSU316, and pHly152 in Southern blot experiments as described previously (5) (Fig. 2). Plasmid pACYC184 contained no sequence homologous to those of the Hly plasmids that could be detected by this technique (5). Thus, all of the homologous bands must represent the homology of the Hly plasmids with the sequence inserted in pACYC184 to produce pSU234. This inserted sequence (located between coordinates 2.5 and 5.5 kb on the pSU234 map of Fig. 1) did not contain sites for either *Eco*RI or *Hin*dIII. Thus, the number of hybridizing bands must represent the minimum number of copies of this sequence in the particular plasmid.

Plasmid pSU233 (the parental plasmid from which the recombinant pSU234 was constructed) showed at least three copies of the sequence, present in *Eco*RI fragments D, F and/or G, and I and *Hin*dIII fragments A, C, and E. The hybridizing signal in *Eco*RI fragment F/G was about

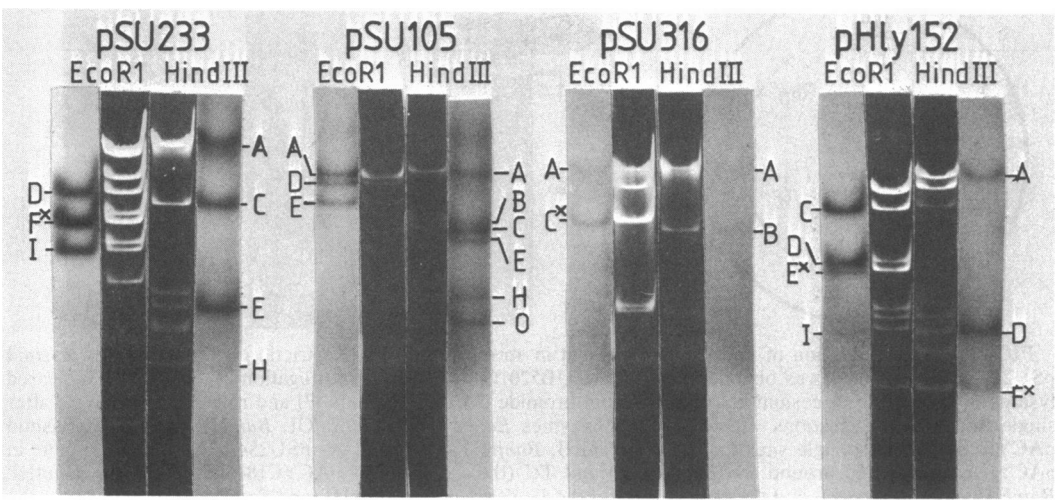

FIG. 2. Hybridization of *Eco*RI and *Hin*dIII restriction fragments from different Hly plasmids with [^{32}P]RNA from plasmid pSU234. [^{32}P]RNA transcribed from pSU234 DNA was obtained by an in vitro reaction with RNA polymerase in the presence of [α-^{32}P]UTP (specific activity, 400 Ci/mmol). The resulting [^{32}P]RNA ($\sim 10^7$ cpm) was hybridized for 42 h at 68°C against a single nitrocellulose filter containing all of the blotted DNA fragments in a total volume of 5 ml as previously described (5). The letters at the side of the photographs refer to the hybridizing fragments. The molecular weights of the *Eco*RI fragments can be found in reference 5. "x" indicates that the hybridizing band is a double band in the gel (i.e., F^x symbolizes hybridization in restriction fragment F and/or G, etc., as described in the text). The weak hybridizing bands were clearly seen in the original autoradiogram.

twice as strong as for the other bands, and there was also weak hybridization against *Hin*dIII-H. Thus, it is possible that pSU233 contains four copies of the sequence.

In vitro RNA transcripts of pSU234 hybridized to *Eco*RI fragments A, D, and E and to *Hin*dIII fragments A, B, C, E, H, and O of pSU105. The intensity of *Hin*dIII-C hybridization was double that of the others. Since the hybridizing *Eco*RI fragments were very large (>10 kb each), we assumed that the number of *Hin*dIII bands that hybridized was a better estimate of the actual number of copies of the sequence, that is, at least six copies. The somewhat weaker intensity of all of the bands in pSU105 was probably just a reflection of the lower amount of plasmid DNA used in these gels.

On the other hand, the two copies of the sequence present in plasmid pSU316 (*Eco*RI fragments A and C and *Hin*dIII fragments A and B) did not hybridize strongly in relation to the amount of DNA used in the experiment. It may be significant that pSU316 comes from a human isolate, whereas pSU233 and pSU105 are of porcine origin (6). Thus, this weaker homology might reflect DNA sequence divergence of the copies of the element present in pSU316 from the ones in the other three plasmids used. The hybridization reactions were carried out under conditions that allowed reassociation to take place between sequences with a maximum of about 15% nucleotide mismatch.

Finally, in vitro RNA transcripts of pSU234 hybridized strongly to *Eco*RI fragments C and D and to *Hin*dIII fragment D and showed, in addition, weak hybridization with fragments *Eco*RI-E/F and -I and *Hin*dIII-A and -F/G of pHly152. Thus, plasmid pHly152 contained at least three or four copies of the repeated sequence.

The ubiquitous presence of this sequence in the Hly plasmids could explain the spread of the α-hemolysin gene among plasmids of different incompatibility groups (6). It also suggests that the large range of homology found among these plasmids (7) is the result of recombinations promoted by these sequences (each single DNA segment bracketed by two ISs should be capable of illegitimate recombination). Although evolutionary divergence from a common ancestor will also produce this result, this seems unlikely to us, since we have compared the basic replicons of the Hly plasmids by Southern hybridization techniques and found that they are not related (J. C. Zabala, F. de la Cruz, and J. M. Ortiz, manuscript in preparation).

In other studies, pSU234 was observed to be transferred by plasmid R388 (4) at a frequency of 5×10^{-7} per Tp^r transconjugant (Zabala et al., unpublished data). This system has been used by a number of workers to detect and analyze transposable elements (15; F. de la Cruz and J. Grinsted, submitted for publication). It is known that R388 cannot conduct pACYC184 by itself (i.e., limit of detection is $<10^{-9}$). Thus, it would appear that the sequence inserted in pACYC184 to form pSU234 is itself able to promote illegitimate recombination events (namely, cointegrate formation with plasmid R388) and hence behaves as an IS element. If the inserted sequence in pSU234 represents a discrete IS element, it is larger than the known IS elements of *E. coli* (IS*1* to IS*5*; 10), IS*10* of Tn*10* (8, 14), and IS*50* of Tn*5* (1, 9) and only comparable to IS*21*, recently found in plasmid R68.45 (16). Furthermore, this IS-Hly appears to be a new IS unit because, as shown in Fig. 1A, it does not contain restriction sites for *Pst*I (as do IS*1*, IS*3*, IS*4*, and IS*21*), *Hin*dIII (as do IS*2*, IS*3*, and IS*50*), or *Eco*RI (as does IS*5*). This new IS element has been assigned the number IS*91* by The Plasmid Reference Center.

We are greatly indebted to John Grinsted for critical reading and correction of the manuscript and to Juan Garcia-Lobo for helpful discussions and assistance in some of the experiments.

J.C.Z. was a recipient of a grant (Beca de Formación del Personal Investigador) from the Spanish Ministry of Education. This work was supported by a grant from the Comisión Asesora de Investigación Científica y Técnica.

LITERATURE CITED

1. **Berg, D. E., C. Egner, B. J. Hirschel, J. Howard, L. Johnsrud, R. A. Jorgensen, and T. D. Tlsty.** 1980. Insertion, excision and inversion of Tn5. Cold Spring Harbor Symp. Quant. Biol. **45**:115–123.
2. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
3. **Clark, A. J., and G. J. Warren.** 1979. Conjugal transmission of plasmids. Annu. Rev. Genet. **13**:99–125.
4. **Datta, N., and R. W. Hedges.** 1972. Trimethoprim resistance conferred by W plasmids in Enterobacteriaceae. J. Gen. Microbiol. **72**:349–356.
5. **de la Cruz, F., D. Muller, J. M. Ortiz, and W. Goebel.** 1980. Hemolysis determinant common to *Escherichia coli* hemolytic plasmids of different incompatibility groups. J. Bacteriol. **143**:825–833.
6. **De la Cruz, F., J. C. Zabala, and J. M. Ortiz.** 1979. Incompatibility among α-hemolytic plasmids studied after inactivation of the α-hemolysin gene by transposition of Tn802. Plasmid **2**:507–519.
7. **De la Cruz, F., J. C. Zabala, and J. M. Ortiz.** 1980. The molecular relatedness among α-hemolytic plasmids from various incompatibility groups. Plasmid **4**:76–81.
8. **Jorgensen, R. A., D. E. Berg, B. Allet, and W. S. Reznikoff.** 1979. Restriction enzyme cleavage map of Tn*10*, a transposon which encodes tetracycline resistance. J. Bacteriol. **137**:681–685.
9. **Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff.** 1979. A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. Mol. Gen. Genet. **177**:65–72.
10. **Kopecko, D. J.** 1980. Specialized genetic recombination systems in bacteria; their involvement in gene expression and evolution. Prog. Mol. Subcell. Biol. **7**:135–234.
11. **Noegel, A., U. Rdest, and W. Goebel.** 1981. Determination

of the functions of hemolytic plasmid pHly152 of *Escherichia coli*. J. Bacteriol. **145**:233–247.
12. **Noegel, A., U. Rdest, W. Springer, and W. Goebel.** 1979. Plasmid cistrons controlling synthesis and excretion of the exotoxin α-hemolysin of *Escherichia coli*. Mol. Gen. Genet. **175**:343–350.
13. **Royer-Pokora, B., and W. Goebel.** 1976. Plasmid controlling synthesis of hemolysin in *Escherichia coli*. II. Polynucleotide sequence relationships among hemolytic plasmids. Mol. Gen. Genet. **144**:177–183.
14. **Sharp, P. A., S. N. Cohen, and N. Davidson.** 1973. Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. II. Structure of drug resistance (R) factors and F factors. J. Mol. Biol. **75**:235–255.
15. **Sherratt, D., A. Arthur, and M. Burke.** 1980. Transposon-specified site-specific recombination systems. Cold Spring Harbor Symp. Quant. Biol. **45**:275–281.
16. **Willetts, N. S., C. Crowther, and B. W. Holloway.** 1981. The insertion sequence IS21 of R68.45 and the molecular basis for mobilization of the bacterial chromosome. Plasmid **6**:30–52.